Title

Distinctive growth requirements and gene expression patterns distinguish progenitor B cells from pre-B cells.

Journal

The Journal of experimental medicine, 177(4)

ISSN

0022-1007

Authors

Faust, EA
Saffran, DC
Toksoz, D
et al.

Publication Date

1993-04-01

DOI

10.1084/jem.177.4.915

Peer reviewed

Distinctive Growth Requirements and Gene Expression Patterns Distinguish Progenitor B Cells from Pre-B Cells

By Elizabeth A. Faust,* Douglas C. Saffran,* Deniz Toksoz,§ David A. Williams,§‖ and Owen N. Witte*‡

*From *The Department of Microbiology and Molecular Genetics, ‡Howard Hughes Medical Institute, University of California, Los Angeles, California 90024; the §Howard Hughes Medical Institute, The Children's Hospital and Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115; and the ‖Howard Hughes Medical Institute, Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana 46202*

Summary

Long-term bone marrow cultures have been useful in determining gene expression patterns in pre-B cells and in the identification of cytokines such as interleukin 7 (IL-7). We have developed a culture system to selectively grow populations of B lineage restricted progenitors (pro-B cells) from murine bone marrow. Pro-B cells do not grow in response to IL-7, Steel locus factor (SLF), or a combination of the two. c-*kit*, the SLF receptor, and the IL-7 receptor are both expressed by pro-B cells, indicating that the lack of response is not simply due to the absence of receptors. Furthermore, SLF is not necessary for the growth of pro-B cells since they could be expanded on a stromal line derived from Steel mice that produces no SLF. IL-7 responsiveness in pre-B cells is associated with an increase in n-*myc* expression and is correlated with immunoglobulin (Ig) gene rearrangements. Although members of the *ets* family of transcription factors and the Pim-1 kinase are expressed by pro-B cells, n-*myc* is not expressed. Pro-B cells maintain Ig genes in the germline configuration, which is correlated with a low level of recombination activating genes 1 and 2 (Rag-1 and 2) mRNA expression, but high expression of sterile μ and terminal deoxynucleotidyl transferase. Pro-B cells are unable to grow separated from the stromal layer by a porous membrane, indicating that stromal contact is required for growth. These results suggest that pro-B cells are dependent on alternative growth signals derived from bone marrow stroma and can be distinguished from pre-B cells by specific patterns of gene expression.

Our goal has been to develop an in vitro system to selectively grow large numbers of primitive B cells in order to identify genes and growth factors that regulate early B cell development. The use of long-term bone marrow culture systems (1) and transformation of pre-B cells by A-MuLV has permitted detailed analysis of pre-B and more mature cells of the B lineage (2–5). In contrast, little is known about the earliest phases of B cell development because of problems in obtaining stable populations of progenitor cells. Stages in the development of B cells within the bone marrow can be defined by Ig gene rearrangements, growth requirements, and the expression of specific genes and surface markers (for a review see reference 2). The progenitor B (pro-B)[1] cell is the most primitive cell of the B lineage. We define it as a B lineage restricted cell that retains Ig genes in the germline configuration and has the capacity to differentiate into mature B cells expressing diverse antigen receptors. Bone marrow sorted on the basis of antigen expression has revealed that pro-B cells express the pan-B cell antigen, B220, and low levels of Thy-1 (6–8).

The earliest stage of B lymphopoiesis that has been extensively characterized is the immature pre-B cell. These cells have rearranged D and J region segments on at least one allele of the Ig H chain locus and express B220. The surrogate L chains λ_5 and $V_{pre\text{-}B}$ and the *mb-1/B29* genes, whose products are essential for the export of IgM to the surface of B cells (9–11), are expressed at this stage. Immature pre-B cells require signals derived from stroma in addition to IL-7 for growth (8, 12).

Immature pre-B cells differentiate into pre-B cells upon productive V-DJ region joining and expression of μ protein in the cytoplasm ($C\mu$). IL-7 stimulates pre-B cells to divide in the absence of stroma (13–15), and IL-7 can synergize with

[1] *Abbreviations used in this paper:* CM, conditioned medium; IGF, insulin-like growth factor; pro, progenitor; SLF, Steel locus factor; TdT, terminal deoxynucleotidyl transferase.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/04/0915/09 $2.00
Volume 177 April 1993 915–923

Steel locus factor (SLF) to provide a potent growth stimulus for these cells (16, 17). Production of functional Ig L chain and surface expression of IgM, IgD, and Ia molecules correlates with the accumulation of B cells in the periphery, and a loss of responsiveness to IL-7 (8).

Several techniques that employ the use of stromal cell lines and exogenous growth factors have been used to grow clonal lines that exhibit characteristics of primitive B cells (16, 18, 19). The phenotypes of these immature B cell clones are quite variable with respect to the expression of the B220, Ly-1, and Mac-1 antigens. This disparity may reflect the outgrowth of unusual cell phenotypes due to the extreme selection of cloning procedures.

In vitro culture systems to grow populations of B lineage cells can be used to avoid clonal variation. Two modifications of long-term bone marrow cultures (1) were made to isolate populations of pro-B cells (20). First, bone marrow cells were infected with a retroviral construct containing the P210 BCR/ABL gene, and second, the cells were plated onto a stromal cell line, called S17. S17 supports the growth of B lineage cells (21) and does not produce detectable levels of IL-7 or its mRNA as assessed by bioassays and the PCR (20, 22). In the absence of IL-7, pre-B cells do not dominate the cultures and clonal populations of pro-B cells with germline Ig genes were expanded (20). One drawback to this system was the expression of the oncogene which could obscure growth factor requirements.

Recently, we described a culture system (23) to grow populations of pro-B cells from murine bone marrow on S17 in the absence of the BCR/ABL oncogene. These cultures offer a reproducible technique for growing highly enriched populations of pro-B cells in a short period of time. In this report, we have used these cells to investigate gene expression and growth factor requirements of pro-B cells. The results of these experiments indicate that pro-B cells can be clearly separated from the pre-B cells stage of lymphopoiesis by their pattern of gene expression and response to specific cytokines. We show that pro-B cells are dependent on stromal-derived signals distinct from IL-7 and SLF. The system utilized here should be useful in identifying signals that are critical for pro-B cell growth.

Materials and Methods

Mice. BALB/cAN mice were bred and maintained in our colony at the University of California, Los Angeles.

Culture Conditions for Pro-B Cells. Initiation of pro-B cell cultures requires two steps: the establishment of semiconfluent S17 monolayers and the plating of BALB/c bone marrow onto the S17. All cells were grown in RPMI 1640 (Gibco BRL, Gaithersburg, MD) medium supplemented with 5% FCS and 50 μM 2-ME. Approximately 5×10^4 S17 cells were plated on 6 cm^2 petri dishes (Corning Inc., Corning, NY) in 5 ml medium and allowed to expand until ~80% confluent (2–3 d). Single cell suspensions of bone marrow isolated from the femurs and tibias of 3–4-wk-old BALB/c mice (24) were washed and plated at 10^6 cells/ml onto the S17 coated dishes (5 ml per dish). Cultures were fed biweekly as described by Whitlock et al. (24). On days 3–4 of each week, 2 ml fresh medium was added per dish, and on day 4 of each week, 75% of the medium from each dish was replaced. After 3–4 wk, pro-B cells were harvested by collecting all medium and then gently washing the S17 layer with cold PBS. Approximately $2–4 \times 10^6$ pro-B cells are recovered per 6 cm^2 dish.

Cultures were also performed by inserting diffusion chambers (Transwells®; Costar Corp., [Cambridge, MA], 0.4-μm pore size; or cell culture inserts Cyclospore; Falcon Labware [Oxnard, CA], 0.45-μm pore size) into the wells of a 24-well dish containing preestablished S17 layers. 10^5 pro-B cells were plated per well.

Cytokines. Recombinant rat-SLF was kindly provided by K. Zsebo and I. McNiece (Amgen Biologicals, Thousand Oaks, CA). Recombinant human-M-CSF was provided by G. Baldwin (Department of Hematology and Oncology, University of California, Los Angeles, CA). The source of IL-7 was an IL-7 containing COS supernatant produced as described (25). Briefly, subconfluent monolayers of COS cells were transfected by a DEAE-dextran-chloroquine method using 15 g of pcDL-SR (IL-7) plasmid. IL-7 containing supernatants were collected 3 d after transfection and titrated on an IL-7–dependent pre-B cell line, clone H (25).

Flow Cytometry. Cells were stained with a variety of murine-specific mAbs. CD45R (anti-B220 mAb; Pharmingen, San Diego, CA) was conjugated to PE. Goat anti–mouse μ mAb conjugated to FITC was used to detect both surface and cytoplasmic expression of Ig μ protein. The anti-c-*kit* biotin–conjugated antibody (2B8) was a gift from K. Ikuta and I. Weissman (Stanford University, Stanford, CA) (26, 27).

To detect surface expression of B220, cells were incubated with the appropriate mAb for 30 min on ice, washed twice with PBS, 2% FCS and 0.2% sodium azide, and fixed in 1% paraformaldehyde. To detect c-*kit* expression, the cells were first incubated with 2B8, and then incubated an additional 30 min on ice with PE–streptavidin before fixation. To detect cytoplasmic μ expression, cells were fixed in 1% paraformaldehyde overnight, incubated with anti-μ mAb for 15 min in PBS, 0.05% Triton X-100, then incubated an additional 15 min in PBS, 0.1% Triton X-100.

All samples were simultaneously analyzed using isotype controls. Antigen expression was measured using a FACScan® flow cytometer and Lysis II software (Becton Dickinson & Co., San Jose, CA).

The fluorokine murine IL-7 biotin kit (R & D Systems, Inc., Minneapolis, MN) was used to detect IL-7 receptors. The protocol provided by the manufacturer was followed. Cells were incubated first with biotin-conjugated recombinant IL-7 and then with PE–streptavidin, and then washed and run on the FACScan® flow cytometer.

Nucleic Acid Analysis. High molecular weight DNA was prepared and analyzed as described (28). Total RNA was prepared by lysing cells in 4 M guanidinium isothiocyanate and spinning the extract through a CsCl cushion (29). All probes were labeled with α-[^{32}P]ATP by random-primed DNA labeling (Boehringer Mannheim Corp., Indianapolis, IN) and had specific activities of ~10^9 cpm/μg of DNA. Descriptions of specific probes and restriction enzyme digests are given in the figure legends.

PCR Analysis. RNA was prepared as above. The PCR strategy used by Pleiman et al. (30) was followed to detect IL-7R mRNA. Sequences of the oligonucleotide primers were as follows: primer 1, 5′ TTACTTCAAAGGCTTCTGGAGC primer 2, 5′ CTGGCTTCAACGCCTTTCACCTCA. The protocols used by Young et al. (25) for generation of cDNA and PCR were followed with the following modification. Primer 1 was used to generate cDNA, the annealing temperature used in the PCR reaction was 60°C, and 30 cycles of PCR were performed. 10% of the reaction product

was separated on a 2% agarose gel and visualized by ethidium bromide staining.

Results

Cells Cultured on S17 Stroma Retain Ig Genes in the Germline Configuration. Large numbers of pro-B cells were produced for phenotypic analysis using the pro-B cell culture system that has been previously described (23). Pro-B cell cultures were initiated by plating murine bone marrow on S17 stromal layers. Cells harvested after 3–4 wk in culture exhibited a monomorphic cellular morphology characteristic of small- and medium-sized lymphocytes. Greater than 70% of the pro-B cells in the population expressed the B220 antigen. The cells were negative for the myeloid surface antigen Mac-1 and for mature T cell antigens (CD3, CD4, and CD8), but expressed low levels of Thy-1. Less than 2% of the cells expressed Cμ, and sIgM was not detected on any cells within the pro-B cell population. These cells could differentiate into mature Ig-secreting B cells in the SCID mouse (23).

To determine the gene rearrangement status of the cells growing on S17, DNAs collected from cells growing in seven individual cultures were subjected to Southern (DNA) blot analysis. The results in Fig. 1 reveal that most of the cells retained IgH genes in the germline configuration. The apparent discrepancy in the migration of the 6.2-kb germline fragment between the pro-B cells and liver (Fig. 1) can be ascribed to a gel artifact. The germline DNA fragment of liver comigrates with that of the pro-B cells on numerous other autoradiograms. We have chosen to present the autoradiogram in Fig. 1 because it contains DNA from seven individual pro-B cells cultures. TCR γ chain genes are also in the germline configuration (data not shown). A subset of the cultured cells (estimated visually at <10%) has begun H chain gene rearrangements (Fig. 1). These rearrangements probably represent D-J joining since the sizes of the DNA fragments correspond to those previously shown to be DJ rearrangements (31–33).

Expression of Genes Associated with the Recombination Machinery. The paucity of rearrangement at the IgH locus raised the possibility that essential components of the recombination machinery were not expressed in pro-B cells. Transcription of the μ locus correlates with accessibility of this locus for recombination (3). After μ transcription, endonucleolytic cleavages are made between coding and recombination signal sequences, followed by exonucleolytic degradation at the coding sequences. The end can then be resynthesized by a template-independent activity, terminal deoxynucleotidyl transferase (TdT), which creates N region sequences at the joints between D_H and J_H or V_H and DJ. Finally, the two coding strands are ligated. The Rag-1 and Rag-2 genes are active in this recombination process (34, 35), however, their precise role is unclear (for a review of V(D)J recombination see reference 36).

Sterile transcripts of the μ locus were present at high levels in the pro-B cells (Fig. 2). This indicated that the lack of recombination in the pro-B cells was not due to inaccessibility of the locus. TdT was also highly expressed in the pro-B cells (Fig. 2). Thus, an absence of TdT could not explain the germline status of the Ig genes in the pro-B cells. However, Rag-1 and Rag-2 mRNA transcripts were detected at very low levels (Fig. 2). Rag expression was detected in the thymus after an 8-h exposure. However, to detect the Rag genes in pro-B cells and bone marrow, a 4-d exposure of the blot was necessary. The level of Rag message in the pro-B cells is comparable with that in bone marrow which consists of >80% myeloid cells that do not express the Rag genes. The germline status of the majority of the IgH genes in the pro-B cells within the population correlates with, and may be a direct reflection of, the low levels of Rag gene expression.

Despite the low frequency of rearrangements, the *B29* gene was highly expressed in the pro-B cells (Fig. 2). The λ_5 gene is also expressed (data not shown). The B29 gene product is essential for the export of IgM to the surface of B cells and the λ_5 gene encodes a surrogate L chain (10). The data presented here indicate that these genes are expressed before Ig gene rearrangements.

Fig. 2 indicates that the level of actin expression is lower in the pro-B cells than in the tissue samples. This may be a result of less pro-B cell RNA on the gel. However, the difference in actin expression may reflect the fact that the

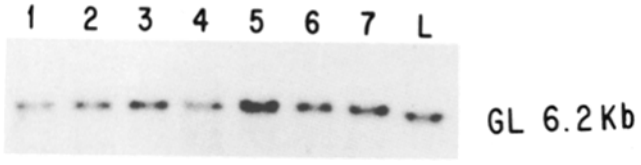

Figure 1. Pro-B cells retain Ig H chain genes in germline configuration. High molecular weight DNA was isolated from seven individual 6-cm² dishes of pro-B cells (lanes *1–7*) 3 wk after establishment of the culture. 10 μg of DNA was digested with EcoRI, electrophoresed through 0.8% agarose gel, transferred to nitrocellulose, and probed with the 1.8-kb H chain J fragment (56). The blot was exposed to film at −70°C for 16 h. BALB/cAN liver DNA (*L*) was used as a germline (*GL*) control.

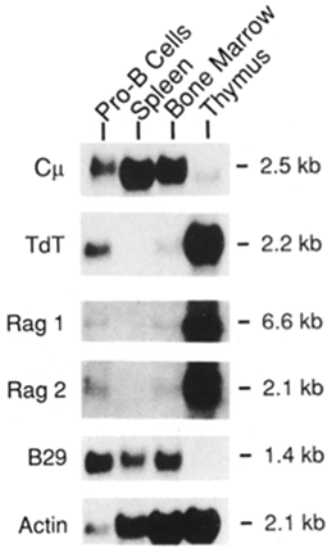

Figure 2. Northern blot analysis of genes associated with recombination. Total cytoplasmic RNA (20 μg) was denatured, fractionated on a formaldehyde-1% agarose gel, transferred to nylon-backed nitrocellulose, blotted with the indicated probes, and exposed to film for the amount of time indicated in parentheses. Probes: 400-bp fragment from the Ig μ C region (1 h) (34); full-length TdT fragment (2 h) (57); 1.3-kb Rag-1 fragment (4 d) (35); full-length Rag-2 fragment (4 d) (35); 1-kb B29 fragment (3 h) (58); and a Puc19 plasmid containing a full-length actin cDNA (16 h). Sizes of transcripts are indicated.

spleen, bone marrow, and thymus samples include connective tissue that is rich in actin, and the pro-B cells are an enriched population of hematopoietic cells with no connective tissue.

Pro-B Cells Express Ets Family Proteins, but Not n-myc. Pro-B cells were analyzed further by Northern blot analysis to monitor genes that regulate early B cell development. The Ets family of putative transcription factors, which includes Ets-1, Pu.1, and Fli-1, has been shown to be expressed in lymphoid tissues and cell lines of mature B lineage cells (37–39). Fig. 3 *A* demonstrates that Ets-1, Pu.1, and Fli-1 are all expressed in pro-B cells, indicating that they may be involved in the transcription of genes expressed at the earliest stages of B cell development.

n-*myc* and c-*myc* belong to a family of cellular proto-oncogenes that are involved in the regulation of transcription (40). c-*myc* is expressed in pre-B cell lines 38B9 and C1 18.81, as well as in the immature pre-B cell line HAFTL-1 and the pro-B cells (Fig. 3). In contrast, n-*myc* is expressed in the pre-B cells lines, but not in HAFTL-1 or the pro-B cells. This implies that n-*myc* is not essential for the growth of pro-B cells, and its expression may mark the pro/pre-B cell junction (41).

The *Pim*-1 proto-oncogene encodes a protein-serine/threonine kinase (42) that is expressed at high levels in the lymphoid tissues and cell lines of both myeloid and lymphoid origin (43). *Pim*-1 is expressed in the two pre-B cell lines tested, the HAFTL-1 cell line, and the pro-B cells (Fig. 3 *b*).

The results presented thus far indicate that pro-B cells can be distinguished from pre-B cells by their germline Ig H chain genes, absence of n-*myc* transcripts, and paucity of Rag-1 and Rag-2 mRNAs.

Neither IL-7 Nor SLF Is Sufficient to Maintain the Growth of Pro-B Cells. Three components have been identified as playing a role in the growth of early B lineage cells: stroma, IL-7, and SLF. The role of soluble IL-7 and SLF in the growth of pro-B cells was assessed by removing them from the stromal layer and replating them at a concentration of 10^5 cells/ml in either medium alone or medium supplemented with growth factors. After 3 d under these conditions, net growth was determined by viable cell counts. The initial seeding concentration was chosen to minimize crossfeeding and to ensure a detectable change in cell number over the assay period.

In each experiment, an IL-7–responsive, pre-B cell line (clone H; 25) and an SLF-responsive line (NSF/60; 44) were included as controls. Clone H increased an average of eightfold (range, 6.1–10.4) when grown under conditions in which 0.2% of

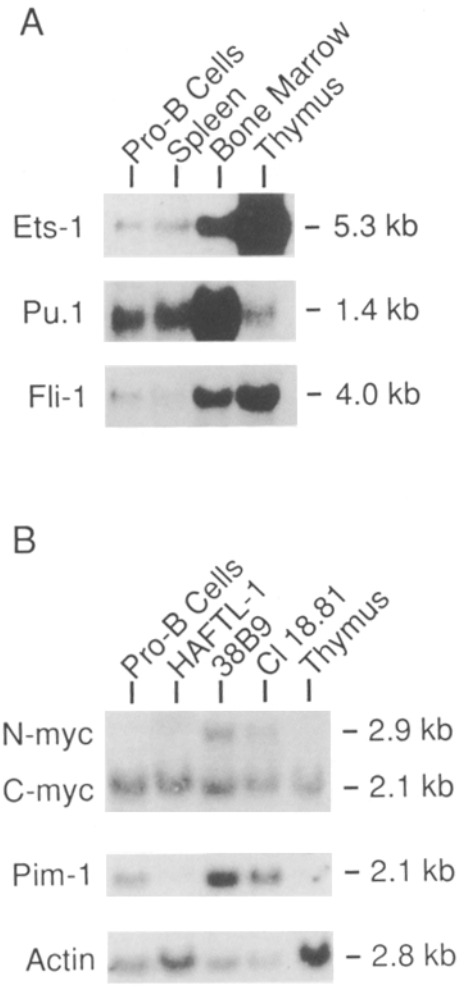

Figure 3. Pro-B cells express the Ets family of transcription factors, Pim-1, and c-*myc*, but not n-*myc*. Blots were prepared as described in Fig 2. (*A*) Probes: full-length cDNA of Ets-1 (8 h) (37); PU.1 (16 h) (38); and Fli-1 (14 h) (59). For a measure of the amount of RNA loaded, an actin probe was used (see Fig. 2). (*B*) Probes: exon 3 of murine n-*myc* which crosshybridizes with c-*myc* (72 h); 1-kb Pim-1 fragment (16 h); and a Puc19 plasmid containing a full-length cDNA of actin (6 h).

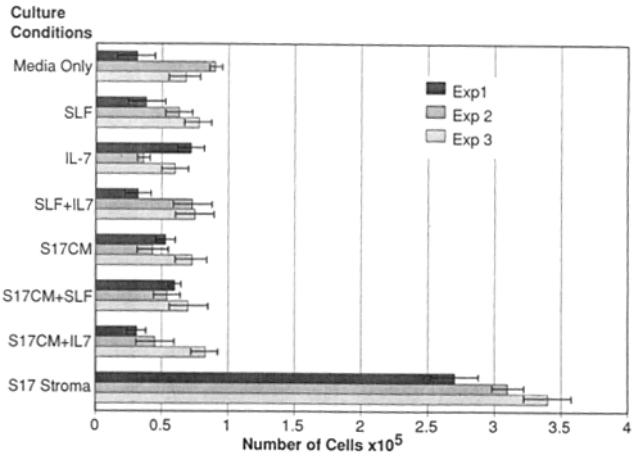

Figure 4. IL-7 and SLF fail to support the growth of pro-B cells. Pro-B cells were harvested after 3 wk in culture, washed, and plated at 10^5 cells/ml in 1 ml on 24-well cluster dishes under the conditions indicated. After 3 d, all cells were harvested and viable cells were enumerated on the basis of trypan blue exclusion. The results of three separate experiments are depicted. Experiment 3 has been normalized to an input cell number of 10^5 from 3×10^5. rSLF (Amgen Biologicals) was used at a concentration of 250 ng/ml. The source of IL-7 was an IL-7–containing COS supernatant (25), which was used at 0.2% of the culture volume. S17CM was collected from a confluent S17 stromal layer 24 h after changing the medium. The results represent the mean and SE (bars) of triplicate samples from each experiment.

the culture volume was IL-7 containing COS supernatant (25). NSF/60 increased an average of 13-fold (range, 9.8–15.0) in medium supplemented with 250 ng/ml SLF (data not shown).

Pro-B cell numbers increased to an average of three times (range, 2.6–3.8) their initial number when grown on the S17 stromal layer. Cells cultured in either 250 ng SLF, 0.2% IL-7, or a combination of the two did not increase in number (Fig. 4). In fact, the number of viable cells recovered after 3 d under these conditions was less than the number initially plated, suggesting that cells had died. Similar results were obtained using various concentrations of SLF (5–500 ng/ml) and IL-7 (0.1–10% IL-7–containing COS supernatant) in seven separate experiments (data not shown).

Pro-B cells were also incubated in medium that had been conditioned by S17 for 24 h (S17 conditioned medium [CM]) to determine if a soluble factor produced by S17 could support growth. Pro-B cells did not usually replicate under these conditions (Fig. 4). However, this result is variable since in two out of seven experiments cells replicated to an average of 1.5 times their original number in S17CM (data not shown).

To determine if either IL-7 or SLF could cooperate with factors secreted by S17 to support the growth of pro-B cells, cells were incubated in S17CM plus either factor. The combination of either IL-7 or SLF with S17CM did not support growth (Fig. 4). These experiments imply that, unlike pre-B cells, the growth of pro-B cells cannot be sustained in IL-7 and SLF.

Pro-B Cells Express c-kit and the IL-7 Receptor. The lack of growth in response to IL-7 and SLF may be due to the absence of the receptors for these factors on the pro-B cells. To determine if c-*kit* (the SLF receptor) was expressed on pro-B cells, cells were incubated with an anti-c-*kit* mAb (2B8; 26) and subjected to flow cytometry. The 2B8 antibody recognizes c-*kit* on IL-3–dependent mast cells derived from +/+ and W/+ mice, but not on mast cells derived from W/W mice (26). Because the W allele has a deletion of the transmembrane domain of c-*kit*, W/W mast cells cannot express c-*kit* on their surface (27). Staining of three populations of pro-B cells revealed that an average of 85% (range, 76–90) of them expressed the SLF receptor (Fig. 5 *A*).

Since no antibodies to the IL-7 receptor (IL-7R) were available, two independent assays were used to demonstrate that the IL-7R is expressed by pro-B cells. First, to detect IL-7R mRNA, PCR was used to amplify a specific fragment after reverse transcription of pro-B cell RNA. As seen in Fig. 5 *B*, IL-7R message was present in pro-B cells and the pre-B cell lines (70Z/3, clone H). IL-7R mRNA was not detected in the myeloid line 32D or the mature B cell line WEHI-231, which served as negative controls. Second, to determine if the cells could specifically bind IL-7, they were incubated with fluorescently labeled IL-7 and then analyzed by flow cytometry. An average of 75% (range, 67–92) of the cells in the pro-B cell population bound IL-7 specifically. This was comparable to the 70Z/3 pre-B cell line in which 90% (range, 87–95) of the cells bound the factor. Taken together, the data in Fig. 5 indicate that pro-B cells do express receptors for both SLF and IL-7. Thus, the lack of response to these factors cannot be simply explained by the absence of receptors.

Stromal Lines that Genetically Lack the SLF Gene Support the Growth of Pro-B Cells. The experiments presented above indicated that neither soluble SLF nor IL-7 provided sufficient signals to support the growth of pro-B cells, but did not establish whether or not these cytokines were necessary for growth. It is unlikely that IL-7 is required for growth since S17 does not produce IL-7 as assessed by bioassays or PCR (20, 22). S17 does produce SLF (22), and thus this factor may be required for the growth of pro-B cells.

To determine if SLF is essential for the growth of pro-B cells, we have utilized a stromal cell line S1/S1 which was derived from long-term marrow cultures of homozygous S1/S1

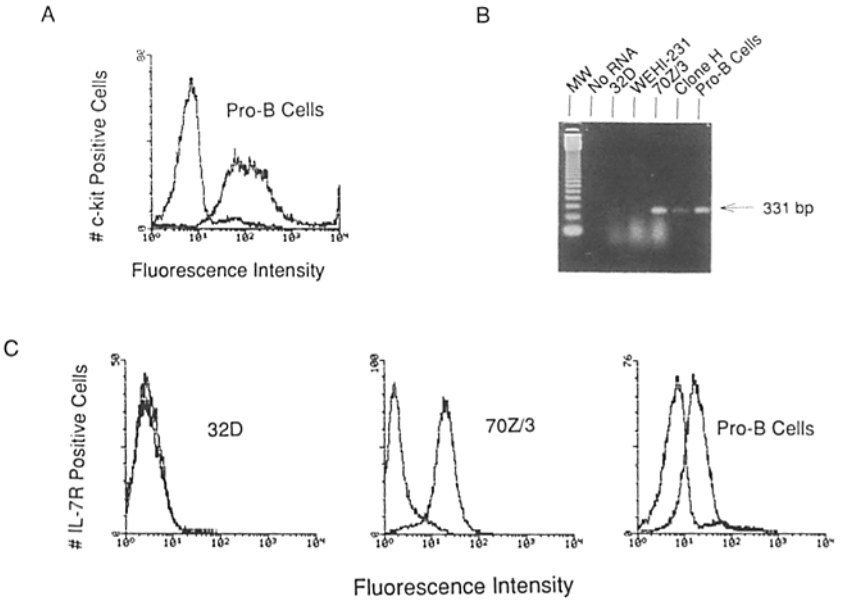

Figure 5. Pro-B cells express the c-*kit* and IL-7 receptors. (*A*) c-*kit* expression on the pro-B cells. Pro-B cells were harvested from S17 and incubated first with biotin-2B8 (anti-*kit*; 44) and then with PE–streptavidin. Background staining was determined by staining cells in only PE–streptavidin. (*B*) IL-7R message detection by PCR. Primers were prepared to detect nucleotide sequences 676–990 of murine IL-7R cDNA (60). mRNA was extracted from the myeloid line 32D, the B cell line WEHI-123, two pre-B cell lines (clone H, 70Z/3), and the pro-B cells. cDNA was made using the downstream primer and reverse transcriptase, and then PCR was performed. Molecular weight markers (*MW*) (123-kb ladder; Gibco BRL). (*C*) Detection of IL-7 R by binding labeled IL-7. Pro-B cells and the cell lines indicated were washed, incubated with an rIL-7–biotin conjugate, and then incubated with streptavidin–PE. Background staining was determined by incubating cells in only PE–streptavidin.

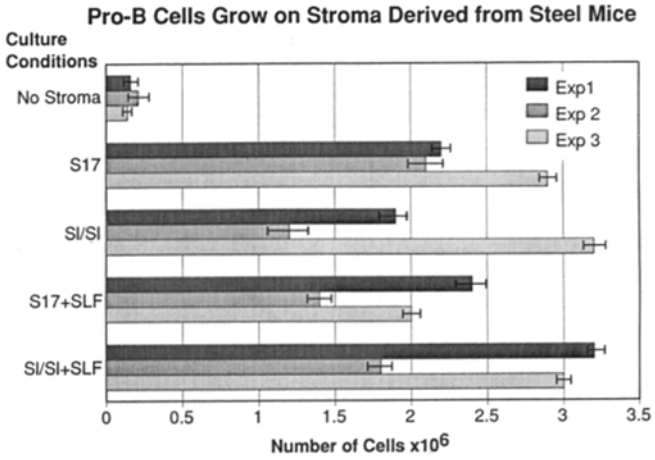

Figure 6. Pro-B cells grow on stroma derived from Steel mice. Pro-B cells were harvested from S17 3–4 wk after establishment of the culture. Cells were washed and 9×10^5 (3×10^5 cells/ml) were plated in 3 ml of medium on the indicated stroma in 6-well cluster dishes. After 4 d, all cells were harvested and the total number of viable cells were counted on the basis of trypan blue exclusion. 300 ng rSLF was added at the initiation of the experiment as indicated. The results represent the mean and SE (bars) of triplicate samples from three different experiments. Experiment 3 has been normalized to an input number of 9×10^5 cells from 10^6.

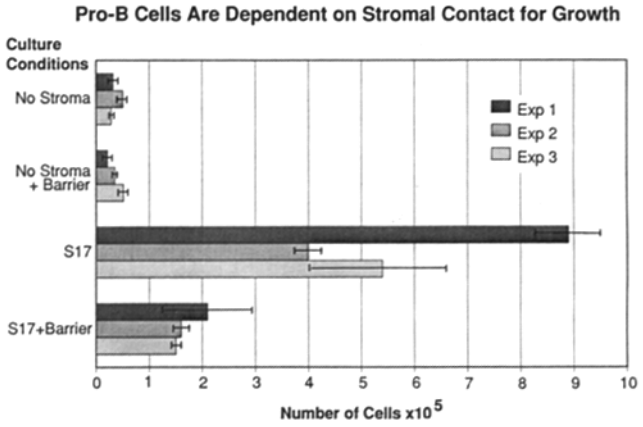

Figure 7. Pro-B cells are dependent on stromal contact for growth. Pro-B cells were harvested from S17 3–4 wk after establishment, washed, and replated at 10^5 cells/ml in 24-well plates in which a subset of the wells had been coated with S17. As indicated, a portion of the wells contained a diffusion chamber (*barrier*) that inhibited pro-B cells from contacting the S17 stromal layer. 6 d after plating, viable cells were counted based upon trypan blue exclusion. The results represent the mean and SE (bars) of triplicate samples from three separate experiments.

null mutant embryos in which the Steel gene was deleted (45, 46). These mice have impaired development of neural crest-derived melanocytes, germ cells, and hematopoietic cells, and do not produce any SLF.

Pro-B cells were carefully removed from the S17 stromal layer by gentle pipetting so that very few S17 cells were removed. They were then washed and replated on either the S1/S1 or S17 stromal line or into tissue culture dishes that contained no stroma. 4 d after transfer, all cells were harvested and counted. No growth was observed in the absence of stroma, indicating that a significant number of S17 cells were not transferred. After incubation on the S1/S1 stroma, the number of cells recovered was an average of sevenfold higher (range, 3.3–11.6) than the initial number of cells (Fig. 6). Pro-B cells grown on S17 increased an average of eightfold (range, 5.5–10.3). Addition of SLF to pro-B cells explanted onto S17 or S1/S1 stroma did not change the growth rate compared with cultures with no exogenous growth factors (Fig. 6). The results of these experiments support the conclusion that SLF is not essential for the growth of pro-B cells.

The Majority of the Pro-B Cells Are Dependent on Stromal Cell Contact for Survival. Pro-B cells appear to be dependent on signals derived from the stromal layer for growth. However, they did not grow well in S17CM (Fig. 4), indicating that they may be dependent on stromal contact, a membrane-bound growth factor, or a labile growth factor produced by the stroma. Previous work with stromal cell cultures has led to the suggestion that the earliest B lineage progenitors are absolutely dependent on signals mediated by direct contact with the layer, whereas latter stages are relatively contact independent (8, 47, 48).

To assess the importance of intercellular contact between pro-B cells and stromal cells, pro-B cells were separated from the stromal layer by a porous membrane. The surface of a 24-well tissue culture plate was coated with S17 stromal cells. Once the stromal layer was confluent, the medium was changed and 24 h later, 10^5 pro-B cells were added either directly on the stroma or into diffusion chambers suspended over the stroma. This protocol allowed the medium in which the cells were plated to be preconditioned by the S17 stroma. Pro-B cells were also plated either directly on wells that contained no stroma, or suspended over plastic in diffusion chambers. All cells were harvested and counted 6 d after transfer. On average, a sixfold increase in cell number was observed (range, 3.5–10.0) in cultures grown in contact with S17 (Fig. 7). When contact with S17 was prohibited by the diffusion chamber, the net increase in cell number was 1.5 (range, −0.4–4.0; Fig. 7). Daily observation of these cultures revealed that after 3 d, >80% of the cells had died. The cells that survived this 3-d period grew rapidly to form small foci. These results indicate that the majority of the cells within the pro-B cell population are dependent on stromal contact for growth, but a subpopulation can respond to a factor secreted by the stromal layer.

Discussion

We have developed a system to reproducibly grow large populations (10^6 cells per 6 cm^2 dish) of pro-B cells that have the capacity to differentiate into mature Ig-secreting B cells in SCID mice (23). These cells were grown in medium supplemented with 5% FCS, but no additional growth factors. Thus, this system provides an opportunity to determine the factors that are important in early B cell growth and differentiation. Using this system, we found that transcription of

sterile μ and TdT precede IgH gene rearrangements and synthesis of mRNA for Rag-1 and Rag-2 during B cell development.

The pro-B cells that populate the cultures described here fall within a fraction of normal bone marrow isolated by Hardy et al. (8) that expressed B220, retained Ig H chain genes in the germline configuration, and was dependent on stromal contact for growth. Two different groups have reported the isolation of B lineage clones that retain Ig genes in the germline configuration (16, 18, 19). The phenotype of these clones did not resemble any of the bone marrow fractions described by Hardy et al. (8) and was variable with respect to surface phenotype. This difference in phenotype may be due to clonal variation.

The kinetics of the establishment of the pro-B cell cultures described here suggested that the pro-B cell populations were derived from many different cells rather than the result of clonal outgrowth. To initiate the cultures, 10^6 bone marrow cells/ml were plated on an S17 stromal layer. After 2 wk in culture, most of the myeloid cells died and multiple foci of lymphopoietic cells grew. These foci replicated so that by 3–4 wk after initiation, the culture was populated with an average of 5×10^5 pro-B cells/ml.

These pro-B cells expressed receptors for IL-7 and SLF, but did not grow in response to these factors in soluble form. c-*kit* is a tyrosine kinase and if the kinase activity is abrogated, then downstream signals are not transduced and there is no response to SLF (49). Immunoprecipitation of c-*kit* from pro-B cells indicated that the full-length protein with normal levels of autokinase activity was produced (Faust, E., and O. Witte, unpublished observation). Therefore, a defect in c-*kit* kinase activity does not explain the lack of response to SLF in pro-B cells.

The lack of growth in response to IL-7 could reflect a block downstream of the IL-7 receptor that renders the cells nonresponsive to IL-7. n-*myc* and c-*myc* expression is induced in pre-B cells in response to IL-7 (50). The absence of n-*myc* in pro-B cells correlated with the nonresponsiveness to IL-7. IL-7 response has also been correlated to Ig gene rearrangements (8). Cells expressing Cμ are the most sensitive to IL-7. The lack of Cμ protein and the paucity of IgH rearrangement in the pro-B cells correlates with the lack of response to IL-7.

McNiece et al. (17) have reported that pro-B cells respond to IL-7, but only when IL-7 is used in combination with SLF. This conclusion is in contrast to our studies and may be a reflection of different target populations. McNiece et al. (17) showed that bone marrow depleted of cells expressing B220 ($B220^-$) differentiated into $B220^+$ cells when cultured in IL-7 and SLF. The $B220^-$ population may have been contaminated with pre-B cells. Billips et al. (22) showed that depletion of bone marrow required several rounds of depletion to remove all $B220^+$ and Cμ^+ pre-B cells. This group (22) went on to show that after several rounds of depletion, $B220^-$ bone marrow did not respond to either IL-7 or SLF.

We have tested various cytokines, in addition to IL-7 and SLF, to identify those that are important in the growth of pro-B cells. IL-3 was tested because two different groups have reported the isolation of IL-3–dependent primitive B lineage clones (16, 18). Pro-B cells did not grow in medium supplemented with up to 20% WEHI-3 supernatant, a source of IL-3 (Faust, E., and O. Witte, unpublished observation). M-CSF was also tested since pro-B cells express mRNA for c-*fms* and because bipotential precursors of B cells and macrophages that respond to M-CSF have been identified by several groups (3, 51, 52). Preliminary results indicated that M-CSF cannot substitute for the S17 stromal layer in supporting the growth of pro-B cells. A recent report demonstrated that insulin-like growth factor 1 (IGF-1), which is produced by S17 stroma, was a differentiation factor for early B cells (53). Attempts to maintain pro-B cells in IGF-1 have failed (Faust, E., and O. Witte, unpublished observation).

The majority of cells within the pro-B cell population required cellular contact with the stromal layer for growth, whereas a small subset could respond to a secreted factor. The critical factor(s) produced by S17 could be produced in a membrane-bound and secreted form similar to M-CSF and SLF (54, 55). The bulk of the cells may respond only to the membrane-bound form, whereas a subpopulation can respond to the secreted form. The pro-B cell cultures described here provide a useful system to identify such factors and examine the role of known or novel genes and molecules in the growth and differentiation of early B cells.

We wish to thank Drs. David Schatz, Randy Wall, Stephen Smale, Jos Domen, Anton Berns, Patricia Ernst, Barbara Graves, and Richard Maki for providing nucleic acid probes.

E. A. Faust is a recipient of National Institutes of Health Clinical and Fundamental Immunology Institutional Training Grant AI-076126. D. C. Saffran is a Fellow of the Leukemia Society. O. N. Witte is an Investigator of the Howard Hughes Medical Institute.

Address correspondence to Dr. Owen W. Witte, Howard Hughes Medical Institute, University of California, Los Angeles, 5-748 MacDonald Building, 10833 Le Conte Avenue, Los Angeles, CA 90024-1662.

Received for publication 24 November 1992 and in revised form 25 December 1992.

References

1. Whitlock, C.A., and O.N. Witte. 1982. Long term culture of B lymphocytes and their precursors from murine bone marrow. *Proc. Natl. Acad. Sci. USA.* 79:3608.
2. Rolink, A., and F. Melchers. 1991. Molecular and cellular origins of B lymphocyte diversity. *Cell.* 66:1081.
3. Alt, F.W., T. Blackwell, and G. Yancopoulos. 1987. Development of the primary antibody repertoire. *Science (Wash. DC).* 238:1079.
4. Whitlock, C., K. Denis, D. Robertson, and O. Witte. 1985. In vitro analysis of murine B-cell development. *Annu. Rev. Immunol.* 3:213.
5. Alt, F., N. Rosenberg, S. Lewis, E. Thomas, and D. Baltimore. 1981. Organization and reorganization of immunoglobulin genes in A-MuLV-transformed cells: rearrangement of heavy but not light chain genes. *Cell.* 27:381.
6. Tidmarsh, G.F., S. Heimfeld, C.A. Whitlock, I.L. Weissman, and C.E. Müller-Seiburg. 1989. Identification of a novel bone marrow-derived B-cell progenitor population that coexpresses B220 and Thy-1 and is highly enriched for Abelson leukemia virus targets. *Mol. Cell. Biol.* 9:2665.
7. Melchers, F., and M. Potter. 1991. Mechanisms of Cell Neoplasia. Workshop at the Basel Institute for Immunology, 14–16 April. 1–19.
8. Hardy, R.R., C.E. Carmack. S.A. Shinton, J.D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213.
9. Sakaguchi, N., S.I. Kashiwamura, M. Kimoto, P. Thaimann, and F. Melchers. 1988. B lymphocyte-lineage-restricted expression of mb-1, a gene with CD3-like structural properties. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3457.
10. Hombach, J., T. Tsubata, L. Leclercq, H. Stappert, and M. Reth. 1990. Molecular components of the B-cell antigen receptor complex of the IgM class. *Nature (Lond.).* 343:760.
11. Bauer, S.R., A. Kudo, and F. Melchers. 1988. Structure and pre-B lymphocyte restricted expression of the VpreB gene in humans and conservation of its structure in other mammalian species. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:111.
12. Hayashi, S.-I., T. Kunisada, M. Ogawa, T. Sudo, H. Kodama, T. Suda, S. Nishikawa, and S.-I. Nichikawa. 1990. Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. *J. Exp. Med.* 171:1683.
13. Namen, A.E., S. Lupton, K. Hjerrild, J. Wignall, D.Y. Mochizuki, A. Schmierer, B. Mosley, C.J. March, D. Urdal, S. Gillis, et al. 1988. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature (Lond.).* 333:571.
14. Lee, G., A.E. Namen, S. Gillis, L.R. Ellingsworth, and P.W. Kincade. 1989. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-β. *J. Immunol.* 142:3875.
15. Sudo, T., M. Ito, Y. Ogawa, M. Iizuka, H. Kodama, T. Kunisada, S.-I. Hayashi, M. Ogawa, K. Sakai, S. Nishikawa, and S.-I. Nishikawa. 1989. Interleukin 7 production and function in stromal cell–dependent B cell development. *J. Exp. Med.* 170:333.
16. Palacios, R., H. Karasuyama, and A. Rolink. 1987. Ly1$^+$ pro-B lymphocyte clone. Phenotype, growth requirements and differentiation *in vitro* and *in vivo*. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3687.
17. McNiece, I.K., K.E. Langley, and K.M. Zsebo. 1991. The role of recombinant stem cell factor in early B cell development. *J. Immunol.* 146:3785.
18. McKearn, J.P., J. McCubrey, and B. Fagg. 1985. Enrichment of hematopoietic precursor cells and cloning of multipotential B-lymphocyte precursors. *Proc. Natl. Acad. Sci. USA.* 82:7414.
19. Palacios, R., and J. Samaridis. 1992. Fetal liver pro-B and pre-B lymphocyte clones: expression of lymphoid-specific genes, surface markers, growth requirements, colonization of the bone marrow, and generation of B lymphocytes in vivo and in vitro. *Mol. Cell. Biol.* 12:518.
20. Scherle, P.A., K. Dorshkind, and O.N. Witte. 1990. Clonal lymphoid progenitor cell lines expressing the BCR/ABL oncogene retain full differentiative function. *Proc. Natl. Acad. Sci. USA.* 87:1908.
21. Collins, L.S., and K. Dorschkind. 1987. A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. *J. Immunol.* 4:1082.
22. Billips, L.G., D. Petitte, K. Dorshkind, R. Narayanan, C.-P. Chiu, and K.S. Landreth. 1992. Differential roles of stromal cells, interleukin-7, and *kit*-ligand in the regulation of B lymphopoiesis. *Blood.* 79:1185.
23. Saffran, D.C., E.A. Faust, and O.N. Witte. 1992. Establishment of a reproducible culture technique for the selective growth of B-cell progenitors. *Curr. Top. Microbiol. Immunol.* 182:34.
24. Whitlock, C.A., D. Robertson, and O.N. Witte. 1984. Murine B cell lymphopoiesis in long term culture. *J. Immunol. Meth.* 67:353.
25. Young, J.C., M.L. Gishizky, and O.N. Witte. 1991. Hyperexpression of interleukin-7 is not necessary or sufficient for transformation of a pre-B lymphoid cell line. *Mol. Cell. Biol.* 11:854.
26. Ikuta, K., and I.L. Weissman. 1992. Evidence that hematopoietic stem cells express mouse c-*kit* but do not depend on steel factor for their generation. *Proc. Natl. Acad. Sci. USA.* 89:1502.
27. Nocka, K., J.C. Tan, E. Chiu, T.Y. Chu, P. Ray, P. Traktman, and P. Besmer. 1990. Molecular bases of dominant negative and loss of function mutations at the murine c-*kit*/white spotting locus: W^{37}, W^{v}, W^{41}, and W. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1805.
28. Schwartz, R.C., L.W. Stanton, S.C. Riley, K.B. Marcu, and O.N. Witte. 1986. Synergism of v-*myc* and v-Ha-*ras* in the in vitro neoplastic progression of murine lymphoid cells. *Mol. Cell. Biol.* 6:3221.
29. Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294.
30. Pleiman, C.M., S.D. Gimpel, L.S. Park, H. Harada, T. Taniguchi, and S.F. Ziegler. 1991. Organization of the murine and human interleukin-7 receptor genes: two mRNAs generated by differential splicing and presence of a type I-interferon-inducible promoter. *Mol. Cell. Biol.* 11:3052.
31. Yancopoulos, G.D., S.V. Desiderio, M. Paskind, J.F. Kearney, D. Baltimore, and F.W. Alt. 1984. Preferential utilization of the most J_H-proximal V_H gene segments in pre-B-cell lines. *Nature (Lond.).* 311:727.
32. Hunt, P., D. Robertson, D. Weiss, D. Rennick, F. Lee, and O.N. Witte. 1987. A single bone marrow-derived stromal cell type supports the in vitro growth of early lymphoid and myeloid cells. *Cell.* 48:997.
33. Tsukada, S., H. Sugiyama, T. Oka, and S. Kishimoto. 1990. Estimation of D segment usage in initial D to J_H joinings in

a murine immature B cell line. *J. Immunol.* 144:4053.
34. Schatz, D.G.., M.A. Oettinger, and D. Baltimore. 1989. The V(D)J recombination activating gene, RAG-1. *Cell.* 59:1035.
35. Oettinger, M.A., D.G. Schatz, C. Gorka, and D. Baltimore. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science (Wash. DC).* 248:1517.
36. Schatz, D.G., M.A. Oettinger, and M.S. Schlissel. 1992. V(D)J recombination: molecular biology and regulation. *Annu. Rev. Immunol.* 10:359.
37. Gunther, C.V., J.A. Nye, R.S. Bryner, and B.J. Graves. 1990. Sequence-specific DNA binding of the proto-oncoprotein *ets-1* defines a transcriptional activator sequence within the long terminal repeat of the Moloney murine sarcoma virus. *Genes & Dev.* 4:667.
38. Klemsz, M.J., S.R. McKercher, A. Celada, C. Van Beveren, and R.A. Maki. 1990. The macrophage and B cell-specific transcription factor PU.1 is related to the *ets* oncogene. *Cell.* 61:113.
39. Ben-David, Y., E.B. Giddens, K. Letwin, and A. Bernstein. 1991. Erythro-leukemia induction by Friend murine leukemia virus: insertional activation of a new member of the *ets* gene family, *Fli-1*, closely linked to c-*ets-1*. *Genes & Dev.* 5:908.
40. Lüscher, B., and R.N. Eisenman. 1990. New light on Myc and Myb. Part I. Myc. *Genes & Dev.* 4:2025.
41. Zimmerman, K.A., G.D. Yancopoulos, R.G. Collum, R.K. Smith, N.E. Kohl, K.A. Denis, M.M. Nau, O.N. Witte, D. Toran-Allerand, C.E. Gee et al. 1986. Differential expression of *myc* family genes during murine development. *Nature (Lond.).* 319:780.
42. Saris, C.J.M., J. Domen, and A. Berns. 1991. The *pim*-1 oncogene encodes two related protein-serine/threonine kinases by alternative initiation at AUG and CUG. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:655.
43. Meeker, T.C., J. Loeb, A. Merrill, and W. Sellers. 1990. The human *Pim-1* gene is selectively transcribed in different hematolymphoid cell lines in spite of a G+C-rich housekeeping promoter. *Mol. Cell. Biol.* 10:1680.
44. Hara, K., T. Suda, J. Suda, M. Eguchi, J.N. Ihle, S. Nagata, Y. Miura, and M. Saito. 1988. Bipotential murine hemopoietic cell line (NFS-60) that is responsive to IL-3, GM-CSF, G-CSF and erythropoietin. *Exp. Hematol.* 16:256.
45. Zsebo, K.M., J. Wypych, I.K. McNiece, H.S. Lu, K.A. Smith, S.B. Karkare, R.K. Sachdev, V.N. Yuschenkoff, N.C. Birkett, L.R. Williams, et al. 1990. Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell.* 63:195.
46. Matsui, Y., D. Toksoz, S. Nishikawa, S.-I. Nishikawa, D. Williams, K. Zsebo, and B.L.M. Hogan. 1991. Effect of *Steel* factor and leukaemia inhibitory factor on murine primordial germ cells in culture. *Nature (Lond.).* 353:750.
47. Kierney, P.C., and K. Dorshkind. 1987. B lymphocyte precursors and myeloid progenitors survive in diffusion chamber culture but B cell differentiation requires close association with stromal cells. *Blood.* 70:1418.
48. Nishikawa, S.-I., M. Ogawa, S. Nishikawa, T. Kunisada, and H. Kodama. 1988. B lymphopoiesis on stromal cell clone: stromal cell clones acting on different stages of B cell differentiation. *Eur. J. Immunol.* 18:1767.
49. Reith, A.D., C. Ellis, S.D. Lyman, D.M. Anderson, D.E. Williams, A. Bernstein, and T. Pawson. 1991. Signal transduction by normal isoforms and *W* mutant variants of the kit receptor tyrosine kinase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2451.
50. Morrow, M.A., G. Lee, S. Gillis, G.D. Yancopoulos, and F.W. Alt. 1992. Interleukin-7 induces N-*myc* and c-*myc* expression in normal precursor B lymphocytes. *Genes & Dev.* 6:61.
51. Davidson, W.F., T.N. Fredrickson, E.K. Rudikoff, R.L. Coffman, J.W. Hartley, and H.C. Morse III. 1984. A unique series of lymphomas related to the Ly-1$^+$ lineage of B-lymphocyte differentiation. *J. Immunol.* 133:744.
52. Cumano, A., C.J. Paige, N.H. Iscove, and G. Brady. 1992. Bipotential precursors of B cells and macrophages in murine fetal liver. *Nature (Lond.).* 356:612.
53. Landrath, K.S., R. Narayanan, and K. Dorshkind. 1992. Insulin-like growth factor-I regulates pro-B cell differentiation. *Blood.* 80:1207.
54. Rettenmier, C.W., and M.F. Roussel. 1988. Differential processing of colony-stimulating factor-1 precursors encoded by two human cDNAs. *Mol. Cell. Biol.* 8:5026.
55. Anderson, D.M., D.E. Williams, R. Tushinski, S. Gimpel, J. Eisenman, L.A. Cannizzaro, M. Aronson, C.M. Croce, K. Huebner, D. Cosman, and S.D. Lyman. 1991. Alternate splicing of mRNAs encoding human mast cell growth factor and localization of the gene to chromosome 12q22-q24. *Cell Growth Differ.* 2:373.
56. Early, P., H. Huang, M. Davis, K. Calame, and L. Hood. 1980. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: V_H, D and J_H. *Cell.* 19:981.
57. Landau, N.R., T.P. St. John, I.L. Weissman, S.C. Wolf, A.E. Silverstone, and D. Baltimore. 1984. Cloning of terminal transferase cDNA by antibody screening. *Proc. Natl. Acad. Sci. USA.* 81:5836.
58. Hermanson, G.G., D. Eisenberg, P.W. Kincade, and R. Wall. 1988. B29: a member of the immunoglobulin gene superfamily exclusively expressed on B lineage cells. *Proc. Natl. Acad. Sci. USA.* 85:6890.
59. Yamanashi, Y., S. Mori, M. Yoshida, T. Kishimoto, K. Inoue, T. Yamamoto, and K. Toyoshima. 1989. Selective expression of a protein-tyrosine kinase, p56^{lyn}, in hematopoietic cells and association with production of human T-cell lymphotropic virus type I. *Proc. Natl. Acad. Sci. USA.* 86:6538.
60. Goodwin, R., D. Friend, S.F. Ziegler, R. Jerzy, B.A. Falk, S. Gimpel, D. Cosman, S.K. Dower, C.J. March, A.E. Namen, and L.S. Park. 1990. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell.* 60:941.